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Award Number: DAMD17-99-1-9361

TITLE: Selective DNA Delivery to Breast Cancer Cells

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REPORT DATE: June 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2000	Annual (1 Jun 99 - 31 May 00)

4. TITLE AND SUBTITLE
Selective DNA Delivery to Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-99-1-9361

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

This grant award seeks to develop innovative new approaches that will permit selective targeting of DNA molecules to breast cancer cells. We hypothesize that one can use specific protein or peptide sequences to direct linked DNA molecules to breast cancer cells; this hypothesis is being experimentally tested.

During the period covered by this annual progress report, we have shown that bacterially-derived recombinant adenovirus penton base protein (ADPB) can be used as a subviral gene delivery system, to target linked plasmid DNA molecules into cultured human cells. We have also shown that peptide phage display technology can be used to identify phage populations which can either bind to, or internalize in, breast cancer cells, and we have constructed T7 bacteriophage particles which express high affinity ligands for cellular HER-2/neu and alphaV beta3 integrin (HER-2/neu and alphaV beta3 integrin are endocytosing cell-surface receptors which are expressed on some breast cancer cells).

The findings obtained to date are generally supportive of our central hypothesis, and they have also provided us with important reagents for further studies. Future experiments will address the ability of phage-selected peptides to mediate gene transfer into breast cancer cells, both in vitro and in vivo.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	49
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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Steve Tait 6/2/00

PI - Signature Date

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INTRODUCTION

A number of gene delivery systems, including virally-based vectors as well as non-viral methods, are presently being explored as potential DNA delivery vehicles for gene- and immuno- therapy of breast cancer. However, currently available DNA delivery vehicles for gene therapy of breast cancer have a very wide host cell range, making it difficult to specifically target them to tumor cells. Therefore, the experiments being performed under the auspices of this grant award seek to develop innovative new approaches that will allow one to selectively target DNA molecules to breast cancer cells. The underlying hypothesis which is being explored is as follows: that one can use specific protein or peptide sequences to selectively target linked DNA molecules to breast cancer cells. This hypothesis is explored by a combination of approaches, including the use of peptide phage display libraries, recombinant adenovirus-derived gene delivery systems, and (ultimately) proof-of-concept experiments in a small animal model for breast cancer.

BODY

Approved Tasks

The following tasks were outlined in the approved statement of work for this grant:

- *Task 1.* Analysis of DNA delivery by adenovirus penton base proteins (months 1 - 12)
- *Task 2.* Application of phage display technology to the identification of breast cancer targeting peptides (months 1 - 15)
- *Task 3.* Analysis of DNA delivery to breast cancer cells by novel peptides (months 16-30)
- *Task 4.* Studies of DNA delivery using an *in vivo* xenograft model for breast cancer (months 25-36)

Research Accomplishments associated with the above tasks

Task 1: Experiments on the analysis of DNA delivery by adenovirus penton base proteins are summarized in the attached manuscript (H. P. Bal, J. Chroboczek, R.W.H. Ruigrok, S. Dewhurst. Adenovirus type 7 penton: Purification of soluble pentamers from *E. coli* and development of an integrin-dependent gene delivery system. Submitted to Eur. J. Biochem., 2000).

Very briefly, the major findings of these experiments have been as follows:
The adenovirus (Ad) type 7 penton base (PB) gene was sequenced and its amino acid composition was deduced from its nucleotide sequence. The penton was expressed in *E. coli* as a soluble C-terminal fusion with GST (GST-Ad7 PB) and was purified by a single-step affinity chromatography. Both GST-Ad7 PB and cleaved (GST-free) Ad7 PB retained the ability to fold into native pentamers as observed by electron microscopy. GST-Ad7 PB was able to bind a synthetic peptide (FK20) derived from the Ad type 7 fiber and retard DNA through a polylysine chain present at the C-terminus of the linker peptide. GST-Ad7 PB was an effective cell transfecting agent when assayed on 293 cells. Transfection was not dependent upon the presence of lysosomotropic agents indicating efficient endosome escape capability. Excess of an RGD-containing peptide derived from Ad7 PB was able to inhibit transfection indicating specific integrin-mediated uptake of the GST-Ad7 PB-FK20-DNA complexes.

Task 2: Experiments aimed at the identification of novel breast cancer targeting peptides have included the following:

Assay and reagent development

Generation of a T7 bacteriophage library displaying random peptides. Random peptide display libraries constructed in the M13 filamentous phage are commercially available, but random peptide display libraries constructed in alternative phage vectors are not. We have therefore constructed a phage display library in the bacteriophage T7, using cloning systems developed by Novagen. This vector system is advantageous because (1) it grows faster than M13, (2) it expresses many more copies of each peptide, on a per-particle basis (415 copies versus 5-10 for the commercially available M13 libraries from New England Biolabs) and (3) because T7 phage has an isocahedral structure of a size that is very similar to that of many mammalian viruses. As a result, T7 phage is likely to be able to subvert pathways which mammalian viruses use to gain entry into mammalian cells -- thereby facilitating our efforts to identify novel peptides which can engage endocytosing receptors on breast cancer cells.

Our T7 library was constructed by inserting the following double-stranded oligonucleotide into prepared T7 vector arms: TGT-$(NNK)_7$-(TGT), where N = any deoxynucleotide and K = T or G. A library comprising 2×10^8 independent clones was derived in this manner, using commercially available packaging systems (Novagen). Twelve clones were then chosen at random for sequence analysis, which revealed that (as expected), each of the clones was different (i.e., independent):

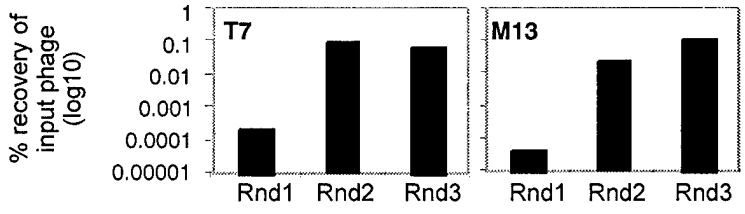
Sequences of randomly selected T7 clones

SAVRKED	*YDGKWA	ESLQLFD	VLSEGTS	ALVEKPR	SSTSPTT
R*PEGA*	GSSNETT	VELHGVG	PPEGRD R	S*AWELV	*RDAAYA

**: stop codon*

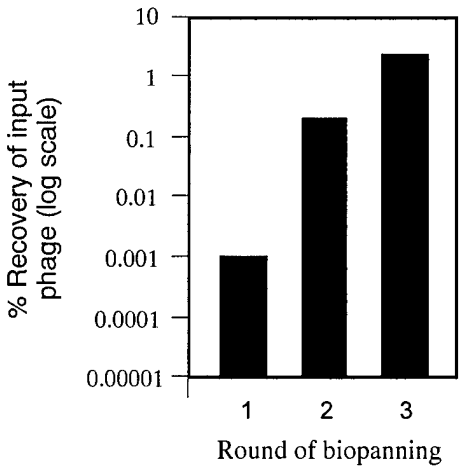
Optimization of ligand-based selection of phage display libraries. We have performed initial biopanning experiments using recombinant mouse CD40 (generous gift of Dr. D. Gray). Two peptide phage display libraries were screened in the experiments – our T7-phage peptide display library (see above) and a commercially available M13-phage library (containing random 12-mer peptide inserts; PhD-12, New England Biolabs). Libraries were reacted with immobilized muCD40 in a 96-well plate for 1 hr at 4°C, after which the well was washed extensively with TBST (Tris-buffered saline with 0.1% Tween-20). Bound phage were then eluted, enumerated by limiting dilution plaque assay, and re-amplified for subsequent rounds of biopanning. The results from this experiment (shown below), emphasize our ability to work with phage display libraries and to carry out iterative cycles of biopanning using this system.

FIGURE 1: Biopanning of a peptide phage display libraries using recombinant murine CD40 (CD40:Ig).



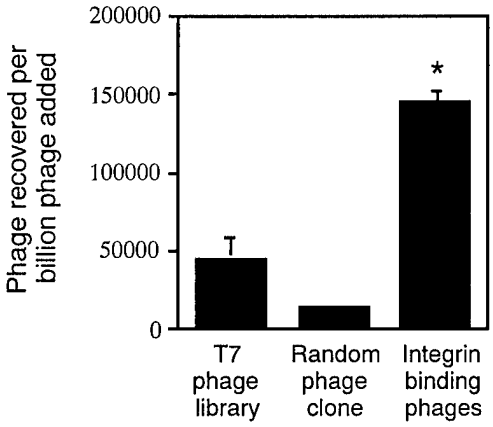
Legend: Two phage display peptide libraries (constructed either in phage T7 or in phage M13) were subjected to 3 rounds of biopanning against muCD40:Ig. The recovery of bound phage is shown for each round of panning (expressed as a percentage of total input phage)

FIGURE 2: AlphaV beta3 biopanning



Recovery of phage populations with an enhanced ability to bind to alphaV beta3 integrin. We have performed biopanning experiments using the T7 bacteriophage peptide display library, in which we have subjected the library to iterative rounds of screening against purified, immobilized alphaV beta3 integrin. The rationale for these studies is that alphaV beta3 is a specific endocytosing cell surface receptor that is expressed on breast cancer cells. These experiments have resulted in the identification of phage populations which have an increased ability to bind to alphaV beta3 integrin (approx. 1000-fold increase in percent recovery of input phage, at the end of three rounds of biopanning; Fig. 2). Sequence analysis of these phage populations is ongoing, as is analysis of cellular internalization of phage selected for the ability to bind to cellular integrins (e.g., see data shown in Fig. 3).

FIGURE 3: Phage Internalization



LEGEND: Equal amounts (10^9 pfu) of each phage population were added to human endothelial cells (HCEC), and phage was allowed to internalize at 37°C. Cell-bound phage was then removed by extensive washing and proteolytic digestion, and internalized phage was rescued by lysis of the cells, and infection of E. coli. Phage titers were then calculated. See text for description of the phage populations that were screened. *: Statistically significant difference from other groups ($p < 0.005$).

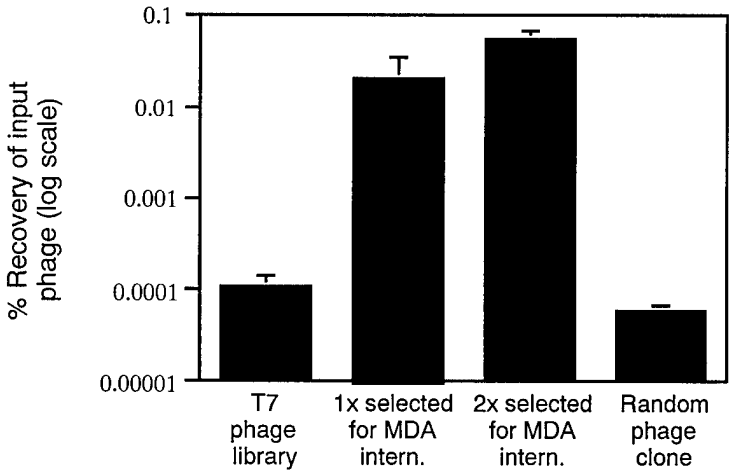
Screening of T7 peptide display phage for the ability to enter human cells. We have developed methods for analysis and recovery of T7 phages which undergo internalization into mammalian cells. To further explore the utility of this technology, we have compared the ability of three different phage populations to undergo internalization into immortalized human endothelial cells (HCEC cell line). For this experiment, we initially examined internalization by (1) unselected peptide-display T7 library (i.e., a diverse phage population, comprising 2×10^8 independent clones), (2) a randomly selected clonal peptide-display T7 phage population, and (3) a peptide-display T7 phage population selected for the ability to bind to purified recombinant $\alpha_v\beta_3$ integrin (selection was for one round only, so this population is expected to be quite diverse and by no means optimized for integrin-binding). The results, which are shown in Fig. 2, reveal that the integrin-selected phage population has a 3-fold enhanced ability to enter HCEC cells, as compared to the starting phage population (the library), and a 10-fold enhanced ability to enter HCEC cells, relative to a negative control phage clone. Thus, even a partial selection for the ability to bind an endocytosing cell surface receptor (such as an integrin) resulted in a significant increase in the ability of T7 phage to enter human cells. This result shows the potential power of this technology, and suggests that selection for cell-internalized phage clones should be quite feasible.

Application of assays to breast cancer cells

Recovery of phage populations with an enhanced ability to enter breast cancer cells. We have used the methods outlined above (Fig. 3) to screen our random peptide display phage T7

library for clones with the ability to undergo internalization into breast cancer cells. These studies have resulted in the identification of phage populations which possess an enhanced ability to undergo internalization into MDA-MB231 cells. Interestingly, phage populations with an enhanced ability to undergo internalization into this cell line did not show an enhanced ability to enter a second breast cancer cell line (SK-BR3; data not shown). This suggests that phage with the ability to enter cells from one particular breast cancer may not necessarily be capable of entering cells from a second, unrelated breast cancer. This could have important implications for the design of breast cancer-targeting strategies.

FIGURE 4: Phage Internalization into MDA-MB231 cells



LEGEND See Fig. 2 and associated text for a description of the methods used. The T7 phage library and random phage clone are the same as those used in Fig. 2, while the phage populations selected for 1 or 2 rounds of internalization into MDA-MB231 cells (MDA intern.) are self-explanatory.

Construction of novel recombinant T7 bacteriophages: We have constructed recombinant T7 phage which express the following molecules: (I) a recently identified high-affinity HER-2/neu binding peptide (FCCGFYACYMDV; [1])and (II) recently described high affinity integrin-binding proteins (snake disintegrins and recombinant derivatives thereof; [2]). The rationale for making these recombinant phage is as follows: HER-2/neu and alphaV beta3 integrins are endocytosing receptors which are expressed on breast cancer cells and/or in the breast cancer environment (i.e., tumor vasculature in the case of alphaV beta3). Thus, recently described peptides and proteins with the ability to bind these molecules at high affinity may have important utility in terms of targeting DNA or other linked molecules (viral or phage vectors) to breast cancer cells. Experiments to test this hypothesis are in progress.

Tasks 3, 4: These tasks remain to be initiated (per the original Statement of Work)

KEY RESEARCH ACCOMPLISHMENTS

- Development of bacterial expression systems for adenovirus penton base protein (ADPB), using glutathione-S-transferase (GST) fusion proteins
- Purification of recombinant ADPB in GST-tagged and GST-free forms
- Analysis of the ability of purified recombinant ADPB to mediate DNA transfer to cultured human cells
- Construction of a random peptide display library in T7 bacteriophage
- Development of protocols to enrich for phage populations capable either of binding to specific cellular receptors, or capable of undergoing internalization in cultured human cells
- Application of these methods to breast cancer cells, and recovery of phage populations with an enhanced ability to enter breast cancer cells
- Application of these methods to a specific endocytosing cell surface receptor expressed on breast cancer cells (alphaV beta3), and recovery of phage populations with an enhanced ability to bind to this molecule
- Construction of recombinant T7 bacteriophages which express (I) a HER-2/neu binding peptide and (II) high affinity integrin-binding proteins (snake disintegrins and recombinant derivatives thereof)

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations:

Manuscript: H. P. Bal, J. Chroboczek, R.W.H. Ruigrok, S. Dewhurst. Adenovirus type 7 penton: Purification of soluble pentamers from *E. coli* and development of an integrin-dependent gene delivery system. Submitted to Eur. J. Biochem., 2000.

Abstract: As above (same title, authors). Oral Presentation at the Third Annual Meeting of the American Society of Gene Therapy, May 31 - June 4, 2000 (Denver, Colorado). Presenter: Dr. H. Bal.

Patents and licenses applied for and/or issued: None

Degrees obtained that are supported by this award: None

Development of cell lines, tissue or serum repositories: None

Informatics such as databases and animal models, etc: GenBank sequence depositions AD001675 & AAF37000

Funding applied for based on work supported by this award: None

Employment or research opportunities applied for and/or received on experiences/training supported by this award: Research training for Ms. Michelle Miller was provided (Ms. Miller is a 1999 college graduate, who has been working on this project as a laboratory technician). It is expected that Ms. Miller will attend graduate school within the next 2-3 years, to obtain her Ph.D. (personal communication from Ms. Miller), and the present research experiences should assist her in that goal. Research training for a summer undergraduate researcher, Ms. Laura McLane, was also provided. Ms. McLane's long-term goals remain uncertain, but may include graduate (Ph.D. or M.D.) school – goals which will be enhanced by her training under this award.

CONCLUSIONS

The conclusions which can be drawn from the first year of our experiments are as follows:

1. Bacterially-derived recombinant adenovirus penton base protein (ADPB) can be used to deliver linked DNA into 293 cells. Delivery into other cell types is being evaluated.

2. Peptide phage display technology can be used to identify phage populations which can either bind to, or internalize in, breast cancer cells. Sequence analysis of selected clones from these phage populations is underway.

3. T7 bacteriophage particles which express high affinity ligands for cellular HER-2/neu or alphaV beta3 integrin can be constructed. Analysis of the ability of these phage to target breast cancer cells is underway.

REFERENCES

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2. Wierzbicka-Patynowski, I., S. Niewiarowski, C. Marcinkiewicz, J. J. Calvete, M. M. Marcinkiewicz, and M. A. McLane. 1999. Structural requirements of echistatin for the recognition of alpha(v)beta(3) and alpha(5)beta(1) integrins. J. Biol. Chem. **274:**37809-37814.

APPENDICES

Manuscript: H. P. Bal, J. Chroboczek, R.W.H. Ruigrok, S. Dewhurst. Adenovirus type 7 penton: Purification of soluble pentamers from *E. coli* and development of an integrin-dependent gene delivery system. Submitted to Eur. J. Biochem., 2000.

Abstract: As above (same title, authors). Oral Presentation at the Third Annual Meeting of the American Society of Gene Therapy, May 31 - June 4, 2000 (Denver, Colorado). Presenter: Dr. H. Bal.

These materials are attached to this report.

APPENDIX MATERIALS

Award Number:	DAMD17-99-1-9361
TITLE:	Selective DNA Delivery to Breast Cancer Cells
PRINCIPAL INVESTIGATOR:	Stephen Dewhurst, Ph.D.
CONTRACTING ORGANIZATION:	University of Rochester Medical Center Rochester, New York 14642
REPORT DATE:	June 2000
TYPE OF REPORT:	Annual

List of Materials Appended

Manuscript: H. P. Bal, J. Chroboczek, R.W.H. Ruigrok, S. Dewhurst. Adenovirus type 7 penton: Purification of soluble pentamers from *E. coli* and development of an integrin-dependent gene delivery system. Submitted to Eur. J. Biochem., 2000.

Abstract: As above (same title, authors). Oral Presentation at the Third Annual Meeting of the American Society of Gene Therapy, May 31 - June 4, 2000 (Denver, Colorado). Presenter: Dr. H. Bal.

ASGT Abstract

H. P. Bal, J. Chroboczek, R.W.H. Ruigrok, S. Dewhurst. Adenovirus type 7 penton: Purification of soluble pentamers from *E. coli* and development of an integrin-dependent gene delivery system. Oral Presentation at the Third Annual Meeting of the American Society of Gene Therapy, May 31 - June 4, 2000 (Denver, Colorado). Presenter: Dr. H. Bal.

Adenoviral gene therapy vectors suffer from the disadvantages of toxicity and immunogenicity associated with the expression of adenoviral gene products from the vector backbone. We report here an alternate strategy for gene delivery that utilizes a single component of the adenoviral type 7 capsid - the penton (Ad 7 PB). The Ad7 PB gene was sequenced and its amino acid composition was deduced from its nucleotide sequence. Ad7 PB exhibited strict conservation of residues essential for pentamerization and fiber-binding. The penton was expressed in E. coli in the soluble form as a C-terminal fusion with GST (GST-Ad7 PB) and was purified by a single-step affinity chromatography. Both GST-Ad7 PB and cleaved (GST-free) Ad7 PB retained the ability to fold into native pentamers as observed by electron microscopy. GST-Ad7 PB was able to bind a synthetic peptide (FK20) derived from the Ad type 7 fiber and retard DNA through a polylysine chain present at the C-terminus of the linker peptide. GST-Ad7 PB was an effective cell transfecting agent when assayed on 293 cells. Transfection was not dependent upon the presence of lysosomotropic agents indicating efficient endosome escape capability. Excess of an RGD-containing peptide derived from Ad7 PB was able to inhibit transfection indicating specific integrin-mediated uptake of the GST-Ad7 PB-FK20-DNA complexe. Integrins have distinct cell-surface expression profiles that can be exploited for delivery to a variety of cell types of lymphoid, hematopoietic, endothelial and epithelial origin, in a specific manner. We propose that Ad7 pentons can be developed into powerful integrin-specific gene delivery agents.

Adenovirus type 7 penton: Purification of soluble pentamers from *E. coli* and development of an integrin-dependent gene delivery system

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Abbreviations: aa, amino acid; Ad, Adenovirus; PB, penton base; Ad7 PB, adenovirus type 7 penton base, GST, glutathione S-transferase; DMEM, Dulbecco's Modified Eagle Medium; RLU, relative light units.

Running title: Adenovirus type 7 penton mediated gene delivery

Keywords: adenovirus; penton base; expression; integrin; gene delivery.

Summary

Adenoviral gene therapy vectors suffer from the disadvantages of toxicity and immunogenicity associated with the expression of adenoviral genes from the vector backbone. We report here an alternative strategy for gene delivery that utilizes a single component of the adenoviral type 7 capsid - the penton base (Ad7 PB). The Ad7 PB gene was sequenced and its amino acid composition was deduced from its nucleotide sequence. The penton was expressed in *E. coli* as a soluble C-terminal fusion with GST (GST-Ad7 PB) and was purified by single-step affinity chromatography. Both GST-Ad7 PB and cleaved (GST-free) Ad7 PB retained the ability to fold into pentamers as observed by electron microscopy. GST-Ad7 PB was able to bind a synthetic peptide (FK20) derived from the Ad type 7 fiber and retard DNA through a polylysine chain present at the C-terminus of this linker peptide. GST-Ad7 PB was an effective cell transfecting agent when assayed on 293 cells. Transfection was not dependent upon the presence of lysosomotropic agents indicating efficient endosome escape capability. Excess of an RGD-containing peptide derived from Ad7 PB was able to inhibit transfection indicating specific integrin-mediated uptake of the GST-Ad7 PB-FK20-DNA complexes. We propose that Ad7 pentons can be developed into integrin-specific gene delivery agents.

Introduction

The initial interaction of adenovirus (Ad) with its host cell is orchestrated by a complex of two oligomeric viral capsid proteins - penton base (PB) and fiber - which together constitute the adenoviral penton. PB protein, present at each of the 12 vertices of the icosahedral Ad capsid, associates with the amino-terminal tail portion of the fiber [9, 13] while the carboxy terminal knob of the fiber, containing the cell-binding domain, projects away from the capsid. During the process of internalization, the Ad fiber first binds to a 46 kDa transmembrane protein that functions as the high-affinity receptor for a number of adenovirus subgroups and the coxsackie B viruses [5, 6, 32]. Ad internalization then proceeds via interaction of an arginine-glycine-aspartate (RGD) sequence on the Ad PB protein with α_v integrins on the cell surface; this interaction promotes virus-receptor endocytosis [2, 10, 16, 17, 19, 20, 34, 35]. Human adenoviruses, probably with the exception of enteric serotypes, use the vitronectin binding integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ to promote virus internalization [2, 34].

Penton base proteins associate in a fiber-independent manner into ~ 300 kDa homopentamers known as pentons. Pentons of some serotypes can also form higher order structures, called dodecahedra, by a combination of 12 pentameric pentons. Dodecahedra are macromolecular complexes with a molecular weight of ~ 3600 kDa. Both pentons and dodecahedra have been shown to interact with cell-surface integrins via RGD motifs [2, 3, 15, 34] which suggests that they might be capable of mediating internalization in the absence of fiber.

Non-viral gene delivery methods based on the native Ad cellular uptake machinery constitute an attractive alternative strategy to Ad vector-based methods. Ad

PB does not influence cellular DNA or protein synthesis when added to cell cultures [18, 34] and may therefore minimize both the immunogenicity and toxicity problems associated with the use of Ad vectors. The development of an integrin-specific gene delivery method may constitute a powerful means for targeting a number of vital body tissues with applications in molecular medicine.

Integrins, a superfamily of α/β heterodimeric cell surface adhesion receptors, are known to mediate cell-cell adhesion and intracellular signaling events that regulate cell survival, proliferation, and migration [1]. Endothelial cells exposed to growth factors, or those undergoing angiogenesis in tumors, wounds, or inflammatory tissue, express high levels of $\alpha_v ß_3$ integrins [7, 14]. Brooks et al. [8] also demonstrated a significant role of $\alpha_v ß_3$ integrins in human angiogenesis and breast tumor growth. For most integrins, the mechanism of ligand recognition depends on one of two short peptide motifs: RGD and LDV [24, 28], both of which are present in penton bases from a number of Ad serotypes, including members of the subgroup B adenoviruses (Ad3, 7 and 11).

We have earlier demonstrated that dodecahedra from Ad serotype 3 can be used for gene delivery [15]. In this strategy, a linker peptide containing the first 20 amino acids of Ad3 fiber was used to combine the cell-targeting activity of the Ad3 dodecahedron with a reporter plasmid construct to achieve intracellular delivery and expression of the reporter gene. The ternary dodecahedron-linker-DNA complex was demonstrated to enter target cells bearing integrin receptors.

Virus-cell interaction mediated through PB is known to vary by serotype [2, 3, 26, 34]. Ad PBs show a high degree of diversity in the amino acid sequence flanking the functionally relevant RGD tripeptide and it is possible that relative efficiencies of

adenovirus PBs to mediate internalization may be a function of these differences. Ad serotype 7, also a member of subgroup B adenoviruses is known to form penton dodecahedra [29]. We therefore set out to sequence the penton base gene from Ad serotype 7. Bacterial expression systems have earlier been used for the expression and purification of PB from Ad serotype 2 and 12 [2, 3]. However, both Ad2 PB and Ad12 PB were found to localize in *E. coli* as insoluble inclusion bodies. Our objective in pursuing this work was two-fold: 1] to devise a simpler expression strategy for PB that yielded soluble protein and preserved the structural and functional integrity of the penton base and, 2] to test the suitability of Ad7 penton as a gene transfer vehicle.

We describe here the sequencing, production from *E. coli* and the structural characterization of Ad serotype 7 penton base. The amino acid composition of Ad7 PB revealed strict conservation of functionally important residues. These included the essential integrin binding sites (RGD and LDV) and residues involved in pentamerization and stable fiber-penton base binding. Ad7 PB was expressed as a fusion with *Schistosoma japonicum* glutathione S-transferase (GST) and could be purified in a soluble form from *E. coli*. The fusion protein (GST-Ad7 PB) and the cleaved (GST free) Ad7 penton base both retained their ability to pentamerize as evidenced by electron microscopy. Although dodecahedra were not detected in the preparation, GST-Ad7 penton base proved to be an effective and integrin-specific cell transfecting agent.

Materials and Methods

Sequencing of Ad7 PB gene.

DNA coding for Ad7 PB was amplified by polymerase chain reaction (PCR) using total Ad7 viral DNA as template. PCR primers were designed on the basis of homology with Ad3 PB and had the following sequence: Ad31 forward primer - AGC GGA TCC AGT ACG ATG AGG AGA CGA GCC GTG; Ad32 reverse primer - AGC AAG CTT TTA GAA AGT GCG GCT TGA AAG AA. The Ad7 PB gene was cloned into pFastBac1 (Life Technologies, Bethesda, MD) as a BamHI - HindIII fragment to produce pAd7PB-FastBac1. The Ad7 PB gene in pAd7PB-FastBac1 was subsequently sequenced by automated procedures.

Construction of expression plasmid pGST-Ad7PB.

The gene for adenovirus type 7 penton base (Ad7 PB) was amplified by PCR from pAd7PB-FastBac1 and cloned as a BamHI-EcoRI fragment in pGEX3X (Glutathione S-transferase Gene Fusion system, Pharmacia). pGEX3X provides for a Factor Xa cleavage site between GST and PB to release GST and aid purification of the fusion partner. The primers used were: 7GX3UP forward primer - CTA TGC GGG ATC CCC ATG AGG AGA CGA GCC GTG CTA and 7GX3DN reverse primer - TGC TGC GAA TTC TTC TTA GAA AGT GCG GCT TGA AAG AAC which incorporated respectively BamHI and EcoRI cloning sites into the Ad7 PB amplicon. The resulting construct, called pGST-Ad7PB, contained the Ad7 PB open reading frame downstream of the *Schistosoma japonicum* glutathione S-transferase (GST) gene. Expression from this vector is driven by the inducible Ptac promoter.

Expression and purification of recombinant GST-Ad7PB.

The expression vector pGST-Ad7PB containing the GST-Ad7PB expression cassette was used to transform *E. coli* BL21 (λDE3). Transformed bacteria were grown in 1 L shake-flask cultures in the presence of 100 μg/ml ampicillin to an optical density of 1.2-1.5 and induced with 0.1 mM (final) IPTG. The GST-Ad7PB fusion protein was purified by affinity chromatography on glutathione agarose (Glutathione Sepharose 4B, Pharmacia), according to the method of Rhim et al. [31]. Briefly, induced total cell pellets from 1 L culture were resuspended in 4 ml EBC buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40) containing 5 mM DTT. Lysates were prepared by homogenization (Polytron homogenizer, Kinematica GmbH) in the presence of 2 mg/ml lysozyme. Lysates were clarified by centrifugation at 10,000 rpm (SS-34 rotor, Sorvall) and supernatants were loaded on a 200 μl glutathione-Sepharose 4B column prewashed with EBC buffer. The column was washed with EBC buffer containing 200 mM NaCl to strip protein bound non-specifically to the column. The fusion protein was eluted with glutathione elution buffer (10 mM GSH in 50 mM Tris-HCl pH 8.0). 200 μl fractions were collected and analyzed on a reducing polyacrylamide gel. Protein concentrations were determined by the Bio-Rad Protein Assay reagent, and peak fractions were stored at 4°C for further analysis.

Factor Xa mediated cleavage of GST tag.

The pGEX3X vector provides for a Factor Xa cleavage site (Ile-Glu-Gly-Arg, with cleavage occurring after Arg) between GST and the fusion partner. Approx. 3 μgs of affinity purified GST-Ad7PB were cleaved at room temperature for various time-points with 1 U of Factor Xa (Sigma) in cleavage buffer consisting of 50 mM Tris-HCl (pH 7.5),

150 mM NaCl and 1 mM $CaCl_2$. The cleavage reaction was terminated by addition of Laemmli buffer and analyzed on a 10% SDS-PAGE gel.

N-terminal sequencing of Ad7PB.

Factor Xa cleaved (GST free) Ad7 PB were blotted on to a PVDF membrane. N-terminal peptide sequencing of the Ad7PB was performed by automated Edman degradation and high pressure liquid chromatography using a Model 476A Protein Sequencer (Applied Biosystems).

Electron Microscopy of GST-Ad 7 PB and Ad7 PB.

GST-Ad7PB and Factor Xa cleaved GST-Ad7PB were dialyzed into water. Protein at a concentration of about 0.1 mg/ml was adsorbed to the clean face of carbon on mica (the carbon-mica interface) and then the carbon film with adsorbed protein was floated onto a solution of 1% SST, a grid was placed on top of the carbon film which was then picked up from the top with a small piece of news paper and air-dried. The samples were photographed in a JEOL 1200 EXII electron microscope at 100 kV under low dose conditions at a nominal magnification of 40,000.

Size analysis of GST-Ad7 PB .

To estimate the size of GST-Ad7 PB, it was subjected to electrophoresis under non-denaturing and non-reducing conditions on a 8% separating gel with a 5% stacking gel. A number of high molecular weight proteins (Pharmacia) were included on the gel to provide a size reference. These were catalase (232 kDa), Ferritin (440 kDa), Thyroglobulin (660 kDa) and Blue Dextran (2 MDa).

DNA retardation by GST-Ad7PB.

A gel shift assay was performed to test whether GST-Ad7PB bound the bifunctional linker FK20 by interacting with its fiber like N-terminus. The linker used was based on adenovirus type 7 fiber and contained the first 20 amino acids from the fiber sequence at the N-terminus, followed by a 20-mer polylysine chain for DNA complexation (K_{20}):

FK20 : m t K R V R- l s d s-F N P V Y P Y-e d e-K_{20}

The amino acids K-R-V-R found near the beginning of the linker correspond to the proposed consensus predicted for the nuclear localization sequence [11, 21], while the amino acids FNPVYPY represent the region in fiber that interacts with the penton base protein [22]. Complexes were prepared by incubating GST-Ad7PB with FK20 at room temperature for 30 minutes. At the end of this time period, 1 μg of DNA was added to allow complexation via the C-terminal polylysine chain. Binding of plasmid by the GST-Ad7PB-FK20 complex was examined by analysis of the electrophoretic mobility of the plasmid DNA, using a 1% TAE-agarose gel.

Adenovirus type 7 PB-mediated gene transfer.

293 cells were grown in DMEM containing 10% fetal bovine serum (FBS). Cells were maintained at 37°C in the presence of 5% CO_2. GST-Ad7PB (5 and 10 μl corresponding to 1 and 2 μg of recombinant protein) was incubated with 1 μg of FK20 peptide for 30 minutes at room temperature. A reporter gene construct containing the firefly luciferase gene downstream of the human cytomegalovirus immediate-early promoter (pCMV-luc) was then added to the PB-FK20 linker complex, and the mixture was incubated for 30 minutes at room temperature.

Cells were plated at a density of 2 x 10^5 per well in a 24 well plate, washed with serum-free medium and exposed to 100 μM chloroquine for 1 hour. Penton base-DNA complexes were then added to cells in triplicate and incubated for 3 hours at 37°C in the presence of 5% CO_2. The penton base-DNA complexes were removed at the end of 3 hours, fresh complete medium was added, and the cells were incubated for an additional 60 hours. At the end of this period, cells were washed with 1x PBS and lysed in 100 μl reporter lysis buffer (Promega). Lysates were clarified by centrifugation at 5000 g and 20 μl of the clarified supernatants were assayed for luciferase activity using a microplate luminometer (LumiCount Model AL10000, Packard) in combination with the Luciferase Assay System (Promega). The results are expressed in relative light units (RLU) per microgram of cellular protein, as estimated using the Bio-Rad Protein Assay reagent.

Results

Ad7 PB gene sequence.

PCR amplification of the Ad7 PB gene from Ad serotype 7 genomic DNA yielded a product of 1632 base pairs in length. The 544-amino acid sequence of the PB gene was deduced from the nucleotide sequence and aligned with Ad2 and Ad3 PBs; Ad7 and Ad3 PB showed 99% identity at the amino acid level (Genbank accessions AAF37000 and S41389, respectively), while Ad7 and Ad2 PB (Genbank accessin P03276) were more divergent (77% predicted amino acid identity). Both integrin-binding motifs, RGD (aa 329 - 331) and LDV (aa 296 - 298) were found to be conserved in Ad7 PB. Also conserved was the RLSNLLG sequence (aa 263 - 269) identified by Hong and Boulanger [22] as a putative fiber-binding domain on Ad2 PB. Sequences in Ad7 PB flanking the RGD domain, a region known to exhibit considerable variation among Ad serotypes [25], were found to be almost identical to those found in Ad3 PB (except for an asparagine residue at position 326 in Ad7 PB versus an aspartate at the corresponding location in Ad3 PB); in contrast, this region of Ad7 PB is quite divergent from its counterpart in Ad2 (as previously noted by Karayan et al. [25]).

Expression of Ad7 PB in *E. coli* and purification by affinity chromatography.

GST-Ad7 PB was expressed in *E. coli* in amounts up to 5 mg/L of culture. The fusion protein was soluble and was purified from clarified bacterial lysates by single step affinity chromatography on a Glutathione Sepharose 4B column. A 90 kDa band corresponding to the combined molecular weights of GST (26 kDa) and Ad7 PB (64 kDa) was detected on a 4-15% SDS-PAGE (Figure 1A, lane 1). This protein reacted with a polyclonal antiserum raised against the Ad3 PB protein (Figure 1B) and with an

anti-GST antibody (data not shown), indicating that an authentic GST-Ad7 PB protein was produced.

Factor Xa cleavage of GST-Ad7PB.

The GST-Ad7 PB fusion could be cleaved with Factor Xa to release the pure penton base from the fusion protein. Factor Xa mediated cleavage of GST-Ad7 PB at the IDGR recognition site between GST and Ad7 PB yielded a band of the expected size (64 kDa), corresponding to the molecular weight of the Ad7PB (Figure 1A, lanes 2-5). Peptide sequencing of the purified, Factor Xa-cleaved Ad7 PB protein confirmed that the purified protein did indeed correspond to Ad7 PB (data not shown), and it also revealed the presence of co-purified 60 kDa GroEL chaperone protein within the protein preparation (this is presumably present at low levels; Fig. 1A).

Structural characterization of Ad7PB proteins.

Electron microscopic analysis of Ad7 PB protein purified from bacteria revealed the presence of pentameric forms of the PB (Figure 2). Five penton base subunits arranged in a symmetrical fashion around a central axis were visible. Pentameric structures of PB were confirmed by cross-linking GST-Ad7 PB with glutaraldehyde and visualizing the higher order forms by PAGE (data not shown). No dodecahedra were detected in the preparations of PB7, either in the cleaved form (without GST) and in the uncleaved form (GST-Ad7 PB); both forms of the protein did however retained the ability to form pentons.

Size analysis of GST-Ad7 PB .

Analysis of the electrophoretic mobility of GST-Ad7 PB on an 8% native polyacrylamide gel revealed that the protein migrated at a position intermediate

between the two highest molecular weight markers (Blue dextran, at 2 mDa, and Thyroglobulin at 660 kDa). GST-Ad7 PB clearly migrated more slowly than either catalase (232 kDa) or Ferritin (440 kDa). This migration pattern is consistent with the folding of the protein into a large macromolecular structure, as would be expected if GST-Ad7 PB were to fold into higher order structures. Based on the size of the monomeric GST-Ad7 PB protein (~90 kDa), the pentamer is expected to have a molecular weight of 450 kDa. This is in keeping with the observed mobility of the protein, upon non-denatured gel electrophoresis (Figure 3), and it further supports our electron microscopy results.

DNA retardation by GST-Ad7 PB.

GST-Ad7 PB was effective in retarding the electrophoretic mobility of plasmid DNA, in the presence of the FK20 linker peptide, in a dose-dependent manner (Figure 4). In the absence of the FK20 linker peptide, the GST-Ad7 PB alone failed to retard plasmid DNA. FK20-dependent binding of plasmid by GST-Ad7 PB was specific to the Ad7 PB moiety since GST alone, either in the presence or absence of FK20, had no effect on the electrophoretic migration of plasmid DNA (Figure 4, lanes 8-10).

GST-Ad7 PB mediated transfection.

The ability of the GST-Ad7 PB fusion protein to mediate gene transfer into cultured human cells was assessed by adding a complex of GST-Ad7 PB plus FK20 preincubated with the luciferase reporter plasmid DNA (GST Ad7PB-FK20-DNA) to 293 cells. Expression of luciferase was observed in 293 cells transfected with the GST Ad7PB-FK20-DNA complex, either in the presence or absence of chloroquine (Figure 5, lanes 4 and 5). A much lower level of luciferase activity was detectable in cells

transfected with complexes containing FK20 peptide plus DNA (i.e., complexes lacking the GST-Ad7 PB protein). This is consistent with our previous finding that an analogous peptide derived from the adenovirus type 2 fiber protein can mediate DNA transfer into some human cell types [36].

While Ad7-derived FK20 peptide could mediate a low -level of DNA uptake into 293 cells, perhaps due to the presence of a tyrosine-based motif (NPXY) that may function as an internalization signal involved in receptor-mediated endocytosis [12, 23, 30], DNA delivery was much more efficient when the GST Ad7PB protein was added to the transfection complex, and transfection also occurred in the absence of chloroquine (Figure 5).

To investigate the specificity of DNA transfection by the GST Ad7PB-FK20-DNA complex, cells were preincubated in the presence of an excess of an RGD-containing peptide derived from the Ad7 PB (NITRGDTYI) prior to the addition of the GST Ad7PB-FK20-DNA complex. This resulted in a strong inhibition of luciferase expression in the transfected cells (Figure 6, lanes 3-5), suggesting that the GST Ad7PB protein facilitates DNA uptake via an integrin-dependent pathway.

Discussion

The adenovirus penton base may be an ideal molecule to exploit as a gene transport vehicle, since it is able to interact in a selective manner with cell-surface integrins to effect internalization. We sequenced the full-length gene coding for Ad serotype 7 PB and found Ad7 PB protein to be 99% identical to the Ad3 PB at the amino acid level. Ad7 PB was also found to retain the integrin-binding peptide motifs present in PB proteins from several other adenovirus serotypes (RGD and LDV), as well as residues involved in penton-fiber binding and pentamerization - factors that were critical for our purpose of designing a DNA delivery reagent.

Expression of Ad7 PB as a C-terminal fusion with *S. japonicum* GST in *E. coli* BL21 cells yielded soluble protein that could be purified from bacterial lysates by a single-step affinity chromatography. To our knowledge, this is the first report of the production of stable Adenoviral pentons from *E. coli*. Bai et al. [2,3] have reported bacterial expression of Ad2 PB and Ad12 PB. In both cases, the PB was found to localize in *E. coli* as insoluble inclusion bodies. Our method represents an improvement over the method reported by Bai and co-workers, both in the soluble nature of expression and also in terms of ease of down-stream processing.

Structural characterization of Ad7 PB by negative stain electron microscopy revealed that the *E. coli*-derived protein was pentameric, like the native form of this protein. Although the penton bases formed from cleaved, GST-free, Ad7 PB were more symmetrical, the GST-tagged protein retained the inherent spontaneous capability of forming pentamers. The fact that a 26 kDa addition (GST) at the N-terminus of penton base did not inhibit pentamer formation indicates that signals for pentamerization may

be located distal to the PB N-terminus. Indeed, most of the residues implicated so far in pentamerization are present at the C-terminus of PB (Y553 and K556) or are located far from the N-terminus (W119) [22]. In addition, the ability of GST-Ad7 PB to bind a fiber-like peptide (FK20), as reflected by the results of our DNA retardation experiments (Figure 4), suggests that the presence of the GST moiety does not interfere with fiber-binding of Ad7 PB. Thus, the conformation of the GST Ad7PB fusion protein appears to be biochemically indistinguishable from that of its native (unfused) counterpart.

N-terminal sequencing of GST-Ad 7 PB also revealed association of the 60 kDa chaperonin GroEL with GST-Ad7 PB. *E. coli* GroEL is known to bind a number of proteins expressed as GST fusions in bacteria [4, 32] and to aid the formation of native structures. Battistoni et al. [4] have demonstrated that *E. coli* chaperonins are able to interact with nascent GST. It is possible that GroEL plays an analogous role in the folding of GST-Ad7 PB and assists the formation of pentamers.

GST Ad7PB was found to transfect 293 cells in an integrin-dependent fashion. This demonstrates that GST Ad7PB is capable of ferrying DNA molecules across cell membranes, and of facilitating their entry into the nucleus. Presumably, the GST Ad7PB-FK20-DNA complex is able to mediate endosome escape in 293 cells, since expression of the luciferase reporter occurred independently of the presence of lysosomotropic agents such as chloroquine (Figure 5).

In previous experiments using recombinant Ad3 PB dodecahedra prepared from insect cell lysates, we obtained levels of reporter gene expression in transfected 293 cells that were approximately 1000-fold higher than the levels reported here, using the *E. coli*-derived GST-Ad7 PB fusion protein [15]. The difference in transfection efficiency

cannot be attributed simply to the presence of the GST moiety, since Factor Xa-cleaved GST-free Ad7 PB protein mediated a similar level of DNA transfer to that obtained with the GST-Ad7 PB fusion (data not shown). Thus, the differences between our current findings and those reported with the insect cell-derived Ad3 base dodecahedra preparations may be due to inherent differences in structural features of the PB versus dodecahedra. GST-Ad7 PB assumes a pentameric configuration that possesses a total of 5 RGD motifs (integrin-binding domains) and 5 binding sites for the FK20 linker peptide. This may explain why base dodecahedra (60 RGD motifs and 60 binding sites for the FK20 peptide) are more efficient gene delivery systems than penton base [15]. We are currently devising strategies to modify *E. coli*-expressed PB in such a manner as to facilitate the formation of dodecahedra.

Overall, the results reported here establish the potential utility of *E. coli*-derived Ad7 PB as a gene delivery agent. Further experiments, including mutagenesis of the residues flanking the RGD motif, will be required in order to optimize this technology, and to exploit its potential for integrin-mediated gene delivery to specific target cell populations [27].

Acknowledgements

The authors thank Drs. Jack Maniloff and George Kampo for assistance with DNA sequence analysis, Karen Jensen for assistance with electron microscopy, and Brian van Wuyckhuyse for assistance with protein sequencing. Sequences described in this paper have been deposited with GenBank (accession numbers AD001675 & AAF37000). The study was funded by NIH grant No. R21 AI44362, and by Award No.

DAMD17-99-1-9361 from the Department of the Army. The U.S. Army Medical Research Acquistion Activity, 820 Chandler Street, Fort Detrick MD 21702-5104 is the awarding and administering acquistion office, and the information content of this article does not necessarily reflect the position or the policy of the Government; no official endorsement should be inferred.

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Figure Legends.

Figure 1: Purification of GST-Ad7PB

(A) Factor Xa mediated cleavage of GST-Ad7PB. GST-Ad7 PB was incubated at room temperature with 1 U of Factor Xa in 1x cleavage buffer. 2 μg equivalents of the protein were removed at various time-points and analyzed by electrophoresis on a 4-15% SDS-polyacrylamide gel. Uncleaved starting material (intact GST-Ad7 PB, approx. 90 kDa) is shown in lane 1, while cleaved Ad7 PB (~65 kDa) can be seen in the other lanes (lanes 2-5 represent 30, 45, 60 and 120 minute digestion with Factor Xa). In all lanes, the presence of free GST protein (~26 kDa) can observed; this is particularly pronounced after Factor Xa cleavage. Numbers on the left represent molecular weight markers (figures in kDa).

(B) Detection of GST-Ad7PB with an anti-Ad 3 PB antibody. Purified, full-length (not Factor Xa-cleaved) recombinant GST-Ad7 PB protein (2 μg) was electrophoresed on a 4-15% SDS-polyacrylamide gel, and a western blot was performed using a polyclonal rabbit antiserum directed against the adenovirus type3 penton base protein (this antiserum was used at a 1:100,000 dilution). Bound antibody was visualized using the ECL™ system (Amersham Pharmacia); numbers on the left represent molecular weight markers (figures in kDa).

Figure 2: Electron microscopic analysis of GST-Ad7 PB and factor Xa cleaved Ad7 PB. Factor Xa cleaved GST-Ad7PB (A) and uncleaved GST-Ad7PB (B) were dialyzed into water, and examined by electron microscopy. Images were photographed

at a nominal magnification of 40,000 with a JEOL 1200 EXII microscope operating at 100 kV under low dose conditions (final magnification shown here is approx. 165,000).

Figure 3: Size analysis of GST-Ad7 PB under native conditions. Purified recombinant GST-Ad7 PB was subjected to electrophoresis under non-denaturing and non-reducing conditions on a polyacrylamide gel with an 8% separating gel and a 5% stacking gel. The gel was stained with Coomassie brilliant blue in order to visualize GST-Ad7 PB and the high molecular weight marker proteins that were also included on the gel (see lane markings); the position of native GST-Ad7 PB is indicated by the arrow.

Figure 4: GST-Ad7PB-mediated DNA retardation via FK20 linker. GST-Ad7 PB or GST alone, was incubated with a luciferase reporter plasmid (pCMV-luc), in the presence and absence of 0.5 μg of the FK20 linker peptide. DNA-protein complexes were analysed by electrophoresis on a 1% agarose gel in 1x TAE. DNA was visualized using ethidium bromide and a photograph of the resulting gel is shown. Lane 1: untreated DNA incubated in buffer (this shows the mobility of free, supercoiled plasmid DNA). Lanes 2, 3 and 4 contain plasmid DNA that was preincubated with 1, 2 and 4 μgs of GST-Ad7 PB (no FK20), while lanes 5, 6 and 7 contain DNA plus FK20 plus increasing amounts of GST-Ad7 PB (1, 2 or 4 μg, respectively). Finally, lanes 8, 9 and 10 contain plasmid DNA plus GST (1, 2 or 4 μg, respectively) plus FK20.

Figure 5: GST-Ad7PB-mediated transfection of 293 cells. 293 cells were incubated with pCMV-luc and luciferase expression was measured in cell lysates collected 60 hours after transfection. Lane 1: pCMV-luc added in the absence of GST-Ad7 PB (control). Lanes 2 and 3: pCMV-luc and FK20 linker peptide added, in the absence (lane 2) or presence (lane 3) of chloroquine. Lanes 4 and 5: pCMV-luc plus FK20 plus GST-Ad7 PB protein added, in the absence (lane 4) or presence (lane 5) of chloroquine. Luciferase activity is expressed in terms of relative light units (RLU) per mg of total protein in the cell lysates. Bars represent the standard error of mean values. The experiment was performed in triplicate and the results shown are representative of 3 experiments which yielded similar data.

Figure 6: Inhibition of GST-Ad7PB-mediated transfection of 293 cells by an RGD-containing peptide. 293 cells were incubated with pCMV-luc and luciferase expression was measured in cell lysates collected 60 hours later. Lane 1: cells treated with pCMV-luc in the absence of GST-Ad7 PB (control). Lanes 2 and 3: Cells exposed to a complex of pCMV-luc and FK20 linker only. Lanes 3 through 5: Cells exposed to a complex of pCMV-luc and FK20 linker plus GST-Ad7 PB protein, in the absence (lane 3) or presence (lanes 4 and 5) of an RGD-containing peptide (NITRGDTYI; added at 10 or 100 μg to lanes 4 and 5, respectively). Luciferase activity is expressed in terms of relative light units (RLU) per mg of total protein in the cell lysates. Bars represent the standard error of mean values. The experiment was performed in triplicate and the results shown are representative of 3 experiments which yielded similar data.

FIGURE 1A

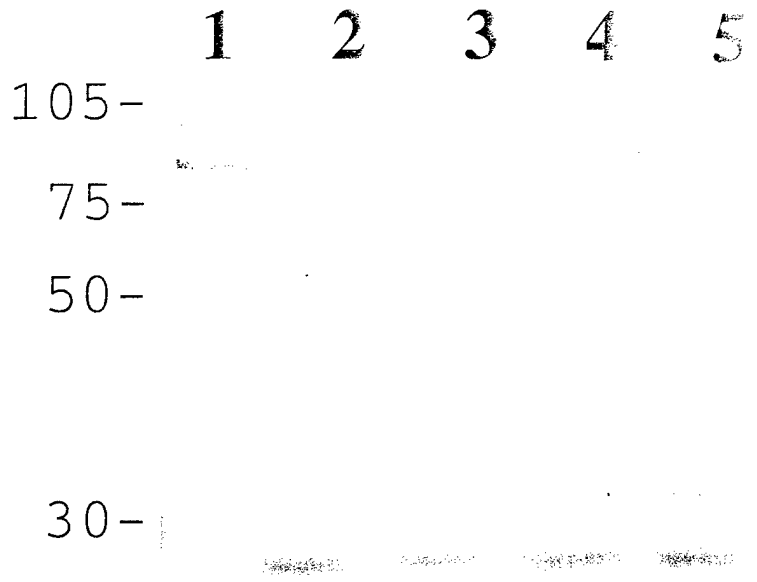


FIGURE 1B

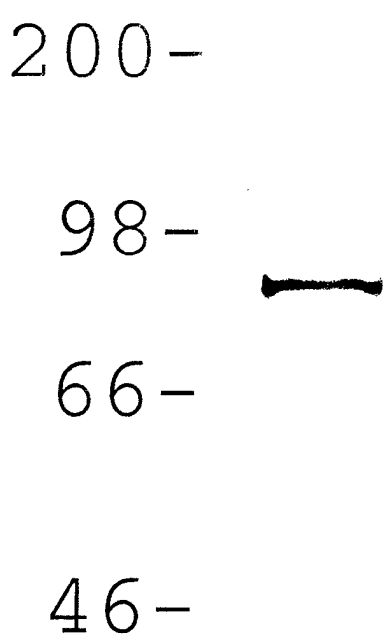


FIGURE 2

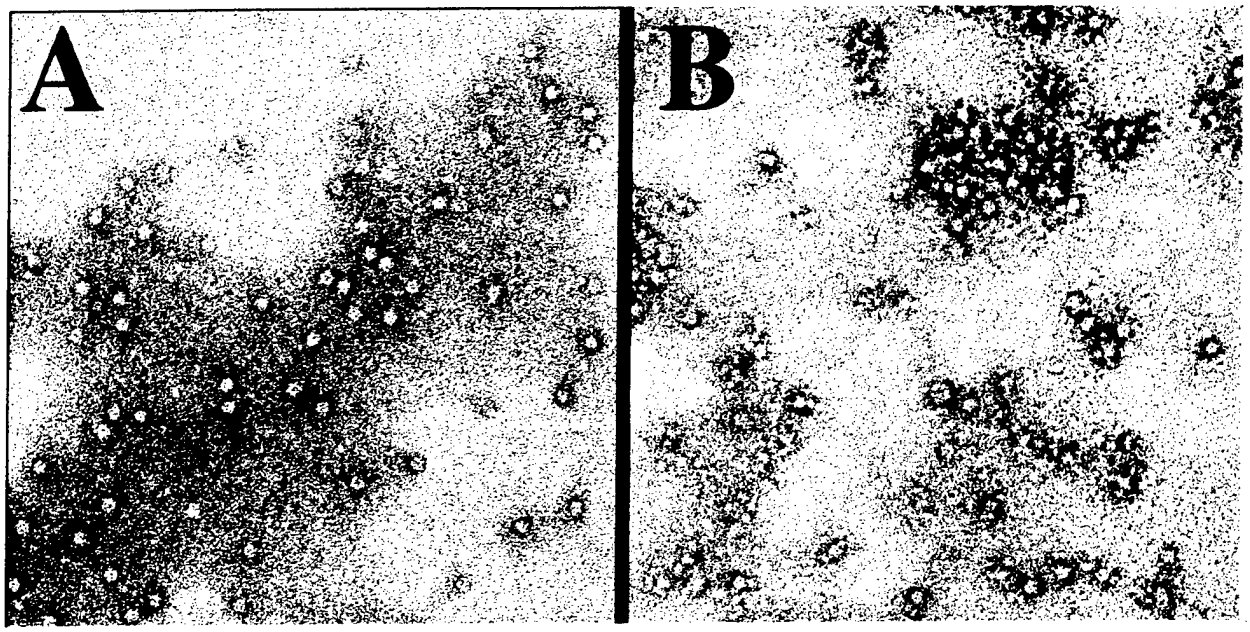


FIGURE 3

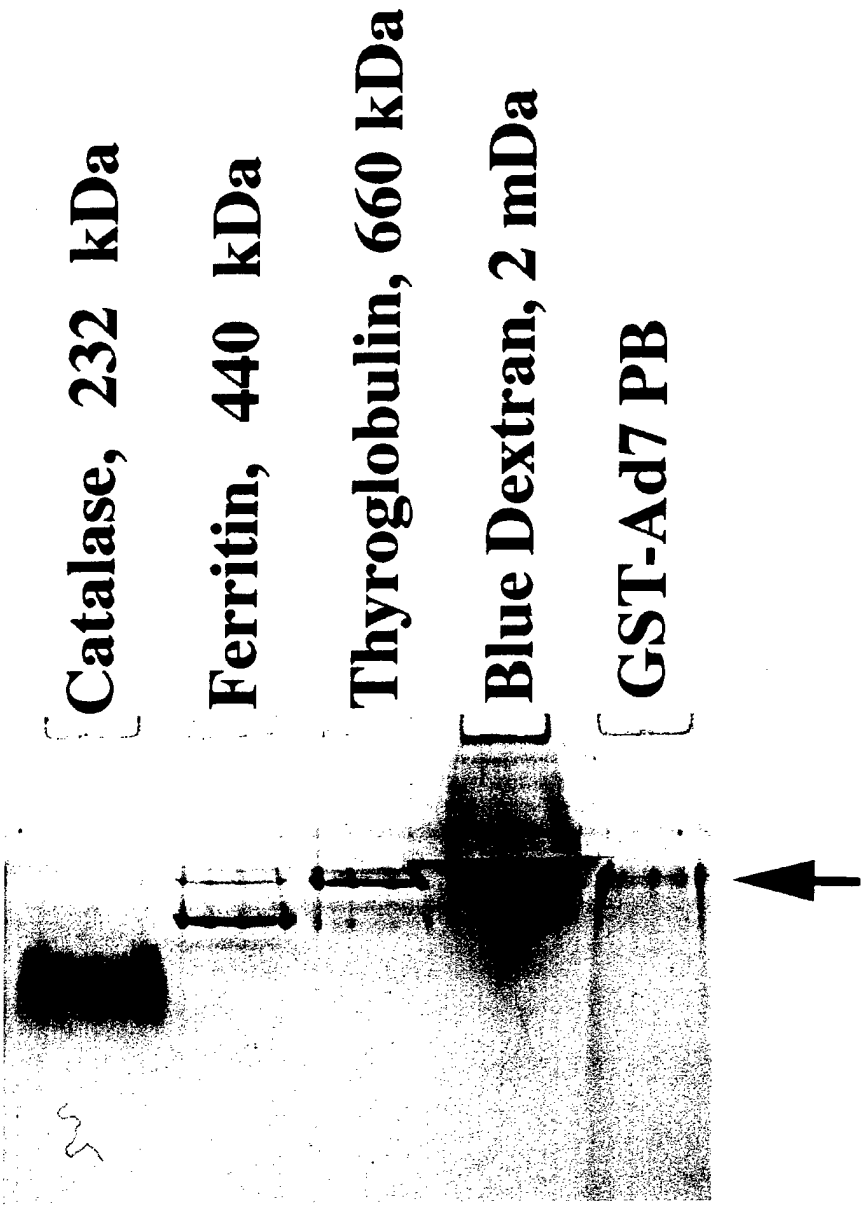

Catalase, 232 kDa
Ferritin, 440 kDa
Thyroglobulin, 660 kDa
Blue Dextran, 2 mDa
GST-Ad7 PB

FIGURE 4

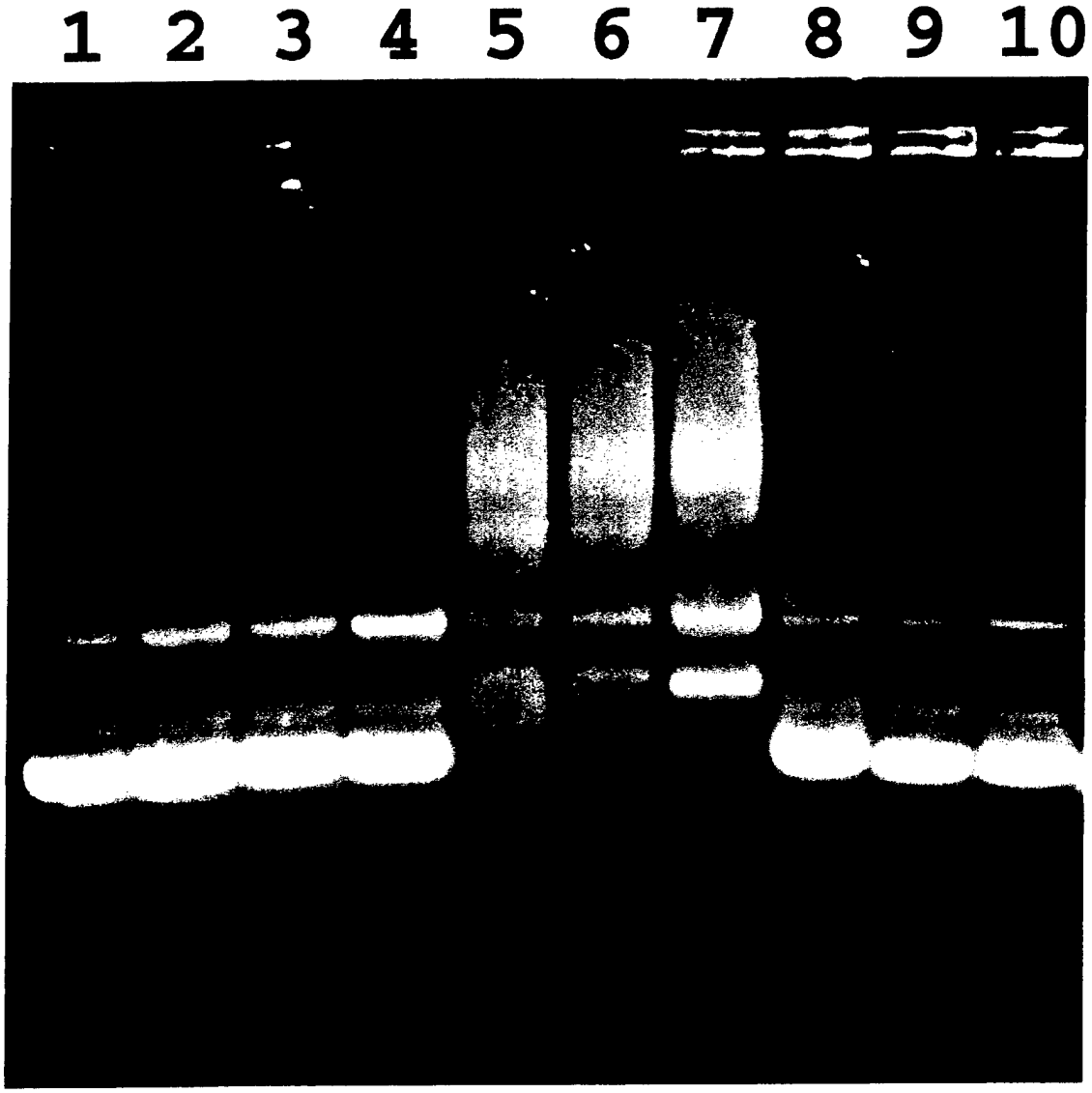


FIGURE 5

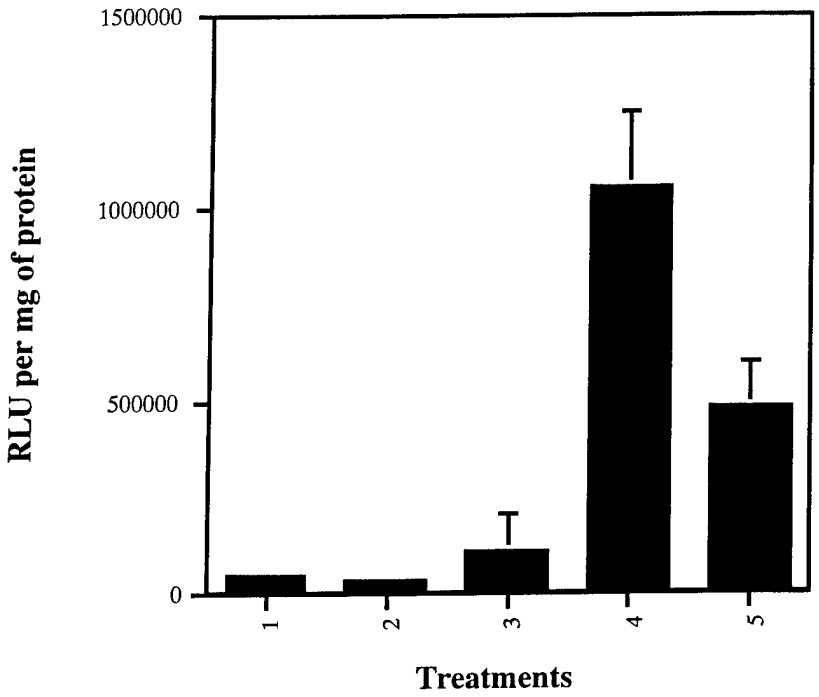
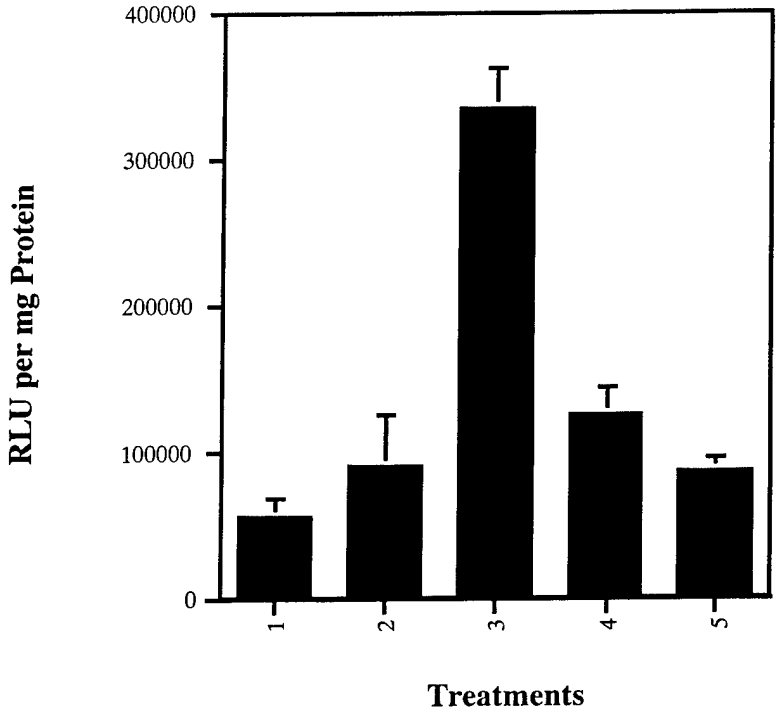

1500000
1000000
500000
0
RLU per mg of protein
1
2
3
4
5
Treatments

FIGURE 6


400000
300000
200000
100000
0
RLU per mg Protein
1
2
3
4
5
Treatments

Bing Chen
Center for Molecular Biology & Gene Therapy
Loma Linda University School of Medicine
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SCHEDULE

Date	Time	What/Where
Thurs., June 1st 2000		Arrive Rochester 3:53pm Taxi to 428 Mt. Vernon (B&B Inn) http://ivillage.bbchannel.com/bbc/p204250.asp
Fri., June 2nd 2000		*Please have breakfast at the B&B (it is included in the cost of accommodation)*
	11:00 AM	Met at B&B by Sanjay Maggirwar
	11:15 AM	Arrival at Dewhurst Lab. (Room 5-8106, UR Med Center)
	11:15-12:00	Meeting with Sanjay Maggirwar
	12:15-1:45	Lunch with grad. students *Joe Sanchez + others* *UR Faculty club*
	2:00-3:00	Seminar (Room 5-8106) *Vaccinia virus based glioma gene therapy: the combination of IL-2, IL-12 and p53*
	3:00-3:45	Meeting with Joe Sanchez, Servio Ramirez *(Ph.D. students, working on projects relating to neuronal apoptosis)*
	3:45-4:15	Brief Tour of URMC – *including Miner library, Core Nucleic Acid Laboratory, Kornberg Building, Flow Cytometry, our lab space plus Chase Bank, Bookstore, Computer Store, Graphics* *(Joe Sanchez/Servio Ramirez)*
	4:15-5:00	Meeting with Ben Segal (Room 1-3007) *(http://www.urmc.rochester.edu/gebs/faculty/benjamin_segal.htm)*
	5:00-5:30 PM	Meeting with Steve Dewhurst, Ph.D.
	7:00	Dinner Steve Dewhurst, Sanjay Maggirwar, Ben Segal Cornhill Cookery (321 Exchange Street, 546-2940)
Sunday, June 4th 2000		Depart Rochester 10:00 AM

SPECIAL SEMINAR

Vaccinia virus based glioma gene therapy: the combination of IL-2, IL-12 and p53

Bing Chen
Loma Linda University School of Medicine

FRIDAY, June 2nd
$2^{00} - 3^{00}$ P.M.
Q-5 Conference Room